The Effects of an Angiogenesis Inhibitor, ATN-224, on the Efficacy of Oncolytic Virus Therapy

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Abstract

Oncolytic viruses (OV) are genetically modified viruses specifically designed to lyse cancerous cells while leaving normal tissue intact. Within the tumor microenvironment, the angiogenic and inflammatory responses following OV inoculation have significantly limited the efficacy of oncolytic herpes simplex virus (oHSV) in clinical trials. Physiological levels of copper support angiogenesis and have been shown to inhibit the infection and replication of wild-type HSV.

Here, we tested if OV replication and cytotoxicity are inhibited by copper and if this inhibition can be rescued by ATN-224, a second generation analog of ammonium tetrathiomolybdate (TM). TM is a copper-chelating agent that is FDA-approved for the treatment of Wilson's disease and is currently under investigation as an anti-angiogenic and anti-neoplastic agent in clinical trials. Under serum concentration of copper, both OV replication and glioma cell killing were significantly inhibited ($P<0.001$). ATN-224 treatment rescued this copper-mediated inhibition of OV replication and glioma cell destruction *in vitro* ($P<0.01$).

The anti-tumor efficacy of this combination therapy was evaluated *in vivo* using two xenograft glioma models. First, mice implanted with subcutaneous U251T3 gliomas were treated with PBS, ATN-224, OV, or OV+ATN224 (n=10). Results indicated that average tumor size in the OV+ATN224 group was significantly smaller compared to OV alone (21.51 vs. 153.93 mm^3, $P=0.0383$). Next, mice implanted with intracranial U87ΔEGFR gliomas were treated as previously (n=8). Kaplan-Meier analysis revealed increased mean survivals for the OV+ATN224 group compared to OV alone (43.875 vs. 24.000 days).

Testing for a biological explanation for this enhanced efficacy, we treated mice daily with ATN-224 or PBS via gavage. OV was administered intravenously, and blood was drawn to detect virus presence. Serum derived from ATN-224-treated mice revealed increased viral presence *in vivo* compared to PBS-treated mouse serum.

Analysis of subcutaneous U251T3 tumors from mice receiving intratumoral OV treatment showed that ATN-224-treated tumors had significantly increased viral replication *in vivo* compared to PBS-treated tumors ($P<0.05$). Similarly, when OV was administered intravenously, ATN-224-treated tumors again displayed increased viral presence compared to PBS-treated tumors. Therefore, the reduced tumor growth and increased survival previously shown *in vivo* might be associated with enhanced viral replication.

Collectively, our results strongly suggest that the co-treatment of ATN-224 with OV can significantly improve the poor efficacy profile of conventional clinical oncolytic virotherapy.

Chapter 1: Introduction

Despite decades of research, over 1500 people die from cancer in the United States every day[1]. Over the years, highly-funded research has led to an understanding of the pathogenesis of cancer and to both non-specific and targeted therapies for treating patients. While these treatments have proved efficacious in some cancer types, there remains a dire need for a novel strategy for a large number of cancers.

Brain Cancer

Although primary brain tumors represent a relatively low proportion of all cancers, the cancer-related morbidity and mortality emphasizes a significant need for attention to discovering effective treatments. Affecting just 48.47 in 1,000,000 children and 24.55 in 100,000 adults, brain tumors constitute less than 2% of all primary malignant cancers[2-3]. Nonetheless, brain tumors are a very lethal, aggressive disease.

The World Health Organization (WHO) classifies primary brain tumors based on their morphological and immunohistochemical features and also on their presumed cell of origin. The WHO classification organizes gliomas, or tumors arising from the glial cells that nourish and protect neurons, into a four-tiered grading scheme that ranges from WHO grade I (least malignant) to WHO grade IV (most malignant). Grade I tumors, primarily pilocytic astrocytoma, have limited potential for malignant progression and can often be cured by surgical resection. Grade II tumors, or diffuse astrocytoma, grow fairly slowly with diffuse infiltration into neighboring brain tissue. Anaplastic astrocytoma, a WHO grade III lesion, demonstrates rapid growth and tends to infiltrate surrounding brain tissue. Glioblastoma multiforme (GBM), or WHO grade IV astrocytoma, is the

most aggressive and most prevalent type of glioma and is characterized by very rapid proliferation and extensive infiltration into surrounding tissue.[4]

Because some non-classic, high grade gliomas, such as anaplastic astrocytoma and GBM, can display little cellular differentiation and can therefore lack visibly distinct pathological features, the diagnosis and prognosis of tumors with such non-classical histology can be variable and also controversial. A more objective approach to glioma classification focuses on analyses of molecular genetics of tumors. For example, one variant of glioblastoma, small-cell GBM[5], is characterized by a gain in chromosome 7 (epidermal growth factor receptor*, EGFR*) and a loss in chromosome 10q (*PTEN*). Gene expression profiles (GEP) have shown a remarkable ability to distinguish histologically ambiguous high-grade gliomas (e.g. GBM and anaplastic oligodendrogliomas) in a clinically relevant manner[6]. In 2009, Gravendeel et al. identified six molecular subtypes of gliomas, including GBM, with distinct prognoses[7]. Gene expression profiling provides a more accurate prognosis for high-grade gliomas than does the traditional histological approach and enables appropriate therapies to be tailored to specific tumor subtypes[8].

The current standard of treatment for GBM is surgical resection, followed by radiation and concurrent daily low-dose temozolomide chemotherapy. This conventional treatment method offers patients with GBM a median survival of just 14.6 months and a dismal 26% two-year survival rate[3]. A potential treatment modality for cancers of this severity lies in oncolytic virus therapy.

Oncolytic Virus (OV) Therapy

Oncolytic viruses (OV) are genetically-modified or naturally-selected strains of viruses that are capable of specifically replicating in and lysing cancerous cells while

avoiding propagation in normal tissue. This is accomplished through the deletion of viral genes that are necessary for its self-propagation. Such an attenuated virus will only be able to propagate in an actively dividing cell that will provide these missing gene products, i.e. a cancer cell. Furthermore, to increase efficacy, these OVs may use tumor-specific promoters to drive propagation-necessary genes that have been deleted[9]. Such a tumor-specific replication of virus will result in lytic destruction of infected malignant cells; normal cells in the brain—which are not actively dividing—will not allow viral DNA replication and will be protected from lysis.

An example of this type of OV is rQnestin34.5. The genome of this oncolytic herpes simplex virus type 1 (oHSV) is characterized by two basic alterations that render the virus tumor-selective in its replication and propagation. One is the disruption of the viral *UL39* locus, which codes for the large subunit of ribonucleotide reductase, a protein critical for the synthesis of DNA precursors[10]. The second is the deletion of both copies of the viral $\gamma_1 34.5$ gene, which encodes a neurovirulent protein product necessary for viral utilization of host cell translational machinery[9]. Although these gene deletions allow this mutant HSV-1 to be more tumor-selective, they can also attenuate the titer of progeny virus in infected cells and thus limit the overall efficacy of OV therapy[11].

To counteract this potential limitation, rQnestin34.5 (isolated from the F strain of HSV-1) contains one copy of the $\gamma_1 34.5$ gene that has been reinserted into the viral genome under the control of a tumor-specific promoter. One gene that is specifically up-regulated in malignant glioma codes for the intermediate filament protein, nestin[12]. While expressed during neuronal embryogenesis and over-expressed in brain cancer,

nestin protein expression is shut off in most regions of the adult brain. In the rQnestin34.5 virus, a nestin promoter element drives the expression of the γ_1*34.5* gene, thus enhancing both the neurovirulent potential and the tumor specificity of this OV within glioma cells[9]. (Figure 1A)

In this study, a second oHSV was utilized based on experimental conditions previously published [13]. Similar to rQnestin34.5, the hrR3 virus (isolated from the KOS strain of HSV-1) also contains a disrupted *UL39* gene locus[10]. However, hrR3 exhibits two functional copies of the γ_1*34.5* gene, and so potentially this virus is not as tumor-selective as is rQnestin34.5. (Figure 1B)

Many types of oncolytic viruses, including oHSV, have been tested in clinical trials in patients with a diverse range of cancers, including gliomas (Table 1). Having been evaluated in phase I, phase II and now phase III clinical trials[14], OV therapy has not revealed evidence of dose-related toxicities, although trial results have been inconclusive with respect to OV effectiveness and patient survival. This observation has encouraged the field to research potential limitations to effective OV therapy.

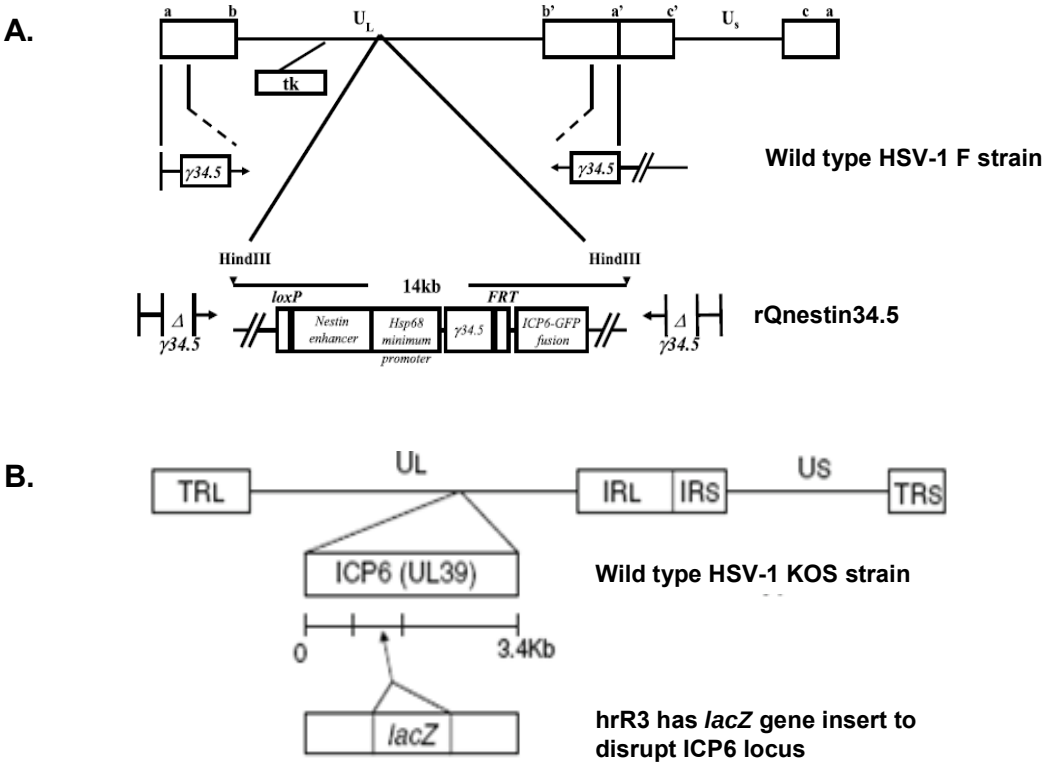


Figure 1. Schematic maps of genomes of rQnestin34.5 and hrR3 oncolytic viruses. A) Genetically modified from HSV-1 F strain (wild type), rQnestin34.5 is characterized by deletions in both copies of the *γ_1 34.5* gene and by insertion of green fluorescent protein (GFP) cDNA into the *UL39* gene (also known as *ICP6*, coding for ribonucleotide reductase). The rQnestin34.5 construct shows the site of recombination of the hybrid promoter (nestin enhancer and hsp68 minimum promoter)-*γ_1 34.5* expression cassette. Overall, rQnestin34.5 contains a disrupted *UL39* locus and one copy of *γ_1 34.5* that is under the control of a nestin promoter element[9]. **B)** hrR3 is genetically engineered from HSV-1 KOS strain (wild type). Here, a *lacZ* gene has been inserted into the *UL39* locus, effectively disrupting the gene encoding viral ribonucleotide reductase. hrR3 contains both functional copies of the *γ_1 34.5* gene[10].

Virus name	Virus Type	Condition	Trial Description	NCT Identifier	Trial Status
GL-ONC1	Vaccina virus	Advanced solid tumors	Phase I, safety/dose toxicity	NCT00794131	Recruiting patients
MV-CEA	Measles virus	Ovarian cancer, peritoneal cavity cancer	Phase I, safety/dose toxicity	NCT00408590	Recruiting patients
REOLYSIN	Reovirus	Malignant glioma	Phase I/II, safety/efficacy	NCT00528684	Completed
G207	Herpes simplex virus	Recurrent malignant glioma	Phase Ib/II, safety/efficacy	NCT00028158	Completed
NDV-HUJ	Newcastle disease virus	Glioblastoma multiforme, sarcoma, neuroblastoma	Phase I/II, efficacy	NCT01174537	Not yet recruiting
OncoVEX(GM-CSF)	Herpes simplex virus	Melanoma	Phase III, safety/efficacy	NCT00769704	Recruiting patients
JX-594	Vaccina virus	Hepatocellular carcinoma	Phase II, safety/efficacy	NCT00554372	Recruiting patients
HSV1716	Herpes simplex virus	Non-CNS solid tumors	Phase I, safety/dose toxicity	NCT00931931	Recruiting patients

Table 1. Oncolytic viruses (OV) in clinical trials. Listed is a sampling of various types of oncolytic viruses that are currently being tested for safety and efficacy in clinical trials against a diverse range of cancers[14]. To date, oncolytic viruses have not shown significant effectiveness with respect to patient survival in many cancer types.

Angiogenesis and the Inflammatory Response

The tumor microenvironment is a key factor to consider when studying the growth and spread of solid tumors[15]. Encompassing the extracellular matrix, blood vessels, immune cells and mediators of angiogenic and inflammatory responses, the tumor microenvironment is embedded in a complex interplay with cancer cells themselves. This introduction will focus on tumor angiogenesis and the inflammatory response in the context of oncolytic virus therapy.

An essential component of tumor survival and progression is angiogenesis, or the generation of new vasculature within the tumor microenvironment. In fact, in the absence of new blood vessel formation, oxygen and nutrients will only be available via diffusion, and tumors will be constrained in size to 2 mm in diameter[16]. Malignant gliomas are characterized by the initiation of blood vessel formation. The pathogenesis of angiogenesis involves dysregulation of the normal homeostasis of angiogenic and anti-angiogenic factors[16-19]. The resulting vasculature features tortuous vessels, exhibiting disruptions in their structural integrity, increased leakiness, uneven focal thickness and arteriovenous shunts not seen in normal brain vasculature[20]. The abnormal circulation that results because of this dysregulated vasculature causes increased interstitial fluid pressure and reduced tumor oxygenation, which together pose a substantial barrier to radiation and chemotherapy[21]. Tumor angiogenesis is closely related to the tumoral inflammatory response, which has significant relevance in the context of oncolytic virus treatment.

A previous study showed that OV treatment both exacerbated the permeability of the tumor vasculature and increased the infiltration of immune cells into the tumor

microenvironment[20]. The increased inflammation post-OV treatment ultimately resulted in rapid clearance of oncolytic virus, thus hindering the efficacy of OV therapy. Interpreting results that show an increased presence of OV in tumors in immunocompromised mice compared to in tumors in immunocompetent mice, it has been suggested that the robust immune response following OV inoculation is, in part, responsible for limiting viral oncolysis[22]. Additional evidence supporting the idea that angiogenesis and inflammation are closely linked in limiting the efficacy of OV therapy can be found in the results of the study by Kurozumi et al. They showed that treating a tumor with an anti-angiogenic agent, cRGD peptide, normalized vascular permeability and reduced OV-induced inflammation, which led to higher viral titers and increased mouse survival[20]. This information suggests that the therapeutic strategy of OV therapy should additionally aim at treating tumor angiogenesis, inflammation, or both.

Importance of Copper

Copper is necessary for normal growth and development, as evidenced by two prominent syndromes, Menkes disease and Wilson's disease, that result when copper is present in deficiency or in excess, respectively. Menkes disease is a deficiency of copper arising from a mutation in a copper-transporting ATPase, which leads to stunted growth, neurological abnormalities and kinky hair[23]. Wilson's disease is an inherited disorder that causes excess accumulation of copper in tissues, resulting in hepatobiliary toxicity, neurological abnormalities and psychiatric manifestations[24]. Copper is relevant to oncolytic virus therapy through its roles in angiogenesis and the inflammatory response.

A key component of blood vessel formation is copper. Copper has been shown to be critical for angiogenesis, as it increases the proliferation and migration of endothelial cells[25]. Copper is an essential co-factor for the secretion of several angiogenic factors. In breast cancer cell lines, treatment with a copper chelator permitted lower levels of pro-angiogenic and pro-inflammatory mediators vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin (IL)-1a, IL-6 and IL-8 to be released. Additionally, rat aortic rings incubated with media from the treated breast cancer cell lines displayed reduced vessel outgrowth compared to aortic rings exposed to media from untreated cells[26]. Copper has also displayed synergistic effects with angiogenin, an inducer of angiogenesis that is secreted by endothelial cells as well as by tumor cells[27]. Evidence has also pointed to a high copper uptake by cancer cells[28], encouraging copper chelation as an anti-cancer therapeutic strategy.

In addition to its role in angiogenesis, copper may have vital functions in the inflammatory and immune processes of the body. Copper is known to have important functions in the development and maintenance of the immune system[29], as it is essential for T-cell proliferation induced by macrophages or by macrophage-mediated cytokines. The stress-induced release pathways of FGF-1 and IL-1a are sensitive to copper-chelating treatment[30], and levels of IL-1b and tumor necrosis factor (TNF)-α (important inflammatory cytokines) are significantly correlated with serum copper concentrations[31].

Interestingly, a unique connection exists between copper and HSV-1. Replication of wild type HSV-1 is known to be inhibited by copper via copper (II)-mediated DNA damage. It is proposed that this damage occurs when Cu(II) ions bind directly to DNA,

especially guanosine residues, and result in breakage of the DNA strand[32]. Due to copper's activity in angiogenesis, the inflammatory process and wild type HSV-1 inhibition, it may be wise to consider copper in the therapeutic strategy for oncolytic virus treatment of cancer.

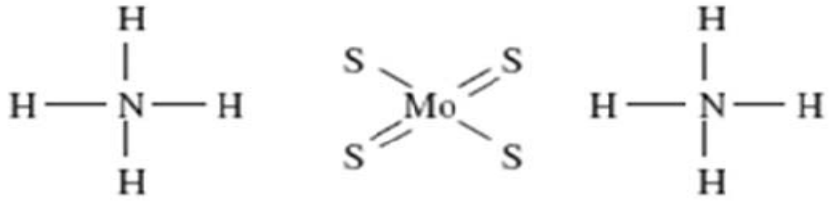
Tetrathiomolybdtate (TM) and ATN-224

Tetrathiomolybdate (TM) is an oral copper-chelating agent that is currently FDA-approved for the treatment of Wilson's disease, showing minimal toxicity in a clinical setting[33]. TM functions by creating a complex with copper and serum albumin, effectively restricting cellular uptake of copper. TM has been shown to be effective for reducing serum copper levels and inhibiting angiogenesis in a variety of studies. For instance, in a murine breast carcinoma model, treatment with TM effectively reduced serum copper levels to 20% of baseline, decreased tumor vascularity and resulted in a 69% reduction in tumor size[26]. Additionally, in a mouse model of multiple sclerosis, TM exhibited strong suppression of inflammatory and immune-related cytokine increases[34]. Inhibition of angiogenesis and tumor metastasis by TM was shown to occur through the suppression of the NFκB signaling cascade, which regulates genes important for angiogenesis, inflammation, invasion and metastasis[35]. TM has also shown synergistic effects with other cancer treatment modalities, including chemotherapy (doxorubicin) and radiation[36].

ATN-224 is a bis-choline salt that is a second generation analog of tetrathiomolybdate (Figure 2). Compared with TM, ATN-224 has superior stability and a faster onset of action[37-38]. It has been proposed that ATN-224 primarily functions by inhibiting the activity of Cu/Zn superoxide dismutase-1 (SOD1) in blood cells[37, 39] and in

endothelial cells[37]. ATN-224 has completed phase I studies in solid tumors[37] and in hematological malignancies and is currently under investigation in several phase II trials as an anti-angiogenic and anti-neoplastic agent in a variety of cancers[14] (Table 2).

TM (ammonium tetrathiomolybdate)



ATN-224 (choline tetrathiomolybdate)

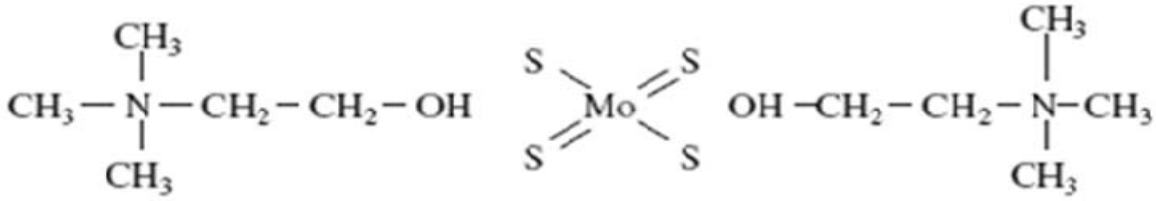


Figure 2. Structures of TM (ammonium tetrathiomolybdate) and ATN-224 (choline tetrathiomolybdate)[38]. ATN-224 is known to have greater stability and a faster onset of action than TM[33, 34].

Drug	Condition	Trial Description	NCT Identifier	Trial Status
TM	Breast cancer	Phase II, safety/efficacy	NCT00195091	Recruiting patients
TM	Lung cancer	Phase I, safety/dose toxicity	NCT00560495	Completed
TM	Psoriasis vulgaris	Phase II, safety/efficacy	NCT00113542	Completed
TM	Esophageal carcinoma	Phase II, safety/efficacy	NCT00176800	Active, not recruiting
ATN-224	Melanoma	Phase II, safety/efficacy	NCT00383851	Unknown
ATN-224	Prostate cancer	Phase II, safety/efficacy	NCT00405574	Unknown

Table 2. Clinical trials of TM and ATN-224. TM and ATN-224 are being tested as anti-angiogenic and anti-neoplastic agents in several studies against multiple diseases, including various types of cancer[14].

Objectives of Study

To date, oncolytic viruses have been tested *in vitro*, in animal models and in several clinical trials. While results *in vitro* and in animal models have shown great efficacy, results from clinical trials have been inconclusive with respect to OV effectiveness and patient survival. Within the tumor microenvironment, the angiogenic and inflammatory responses that follow OV inoculation have been demonstrated to significantly limit OV efficacy. Kurozumi et al. showed that treating a tumor with an anti-angiogenic agent (cRGD peptide) normalized vascular permeability, which suggests the potential for greater viral replication. This study also showed in a rat glioma model that pre-treatment with cRGD and subsequent OV infection significantly increased survival versus treatment with OV alone[20]. By combining ATN-224 treatment with OV therapy, we hope to inhibit angiogenesis (and subsequently inhibit tumoral inflammation) and to increase viral replication in order to enhance the efficacy of OV therapy to ultimately improve survival of patients with glioblastoma.

Specifically, we hypothesize that ATN-224 will improve the efficacy of oncolytic virus therapy against malignant glioma by reducing angiogenesis and inflammation in the tumor microenvironment, thus enhancing the ability of OV to replicate and lyse glioma cells (Figure 3).

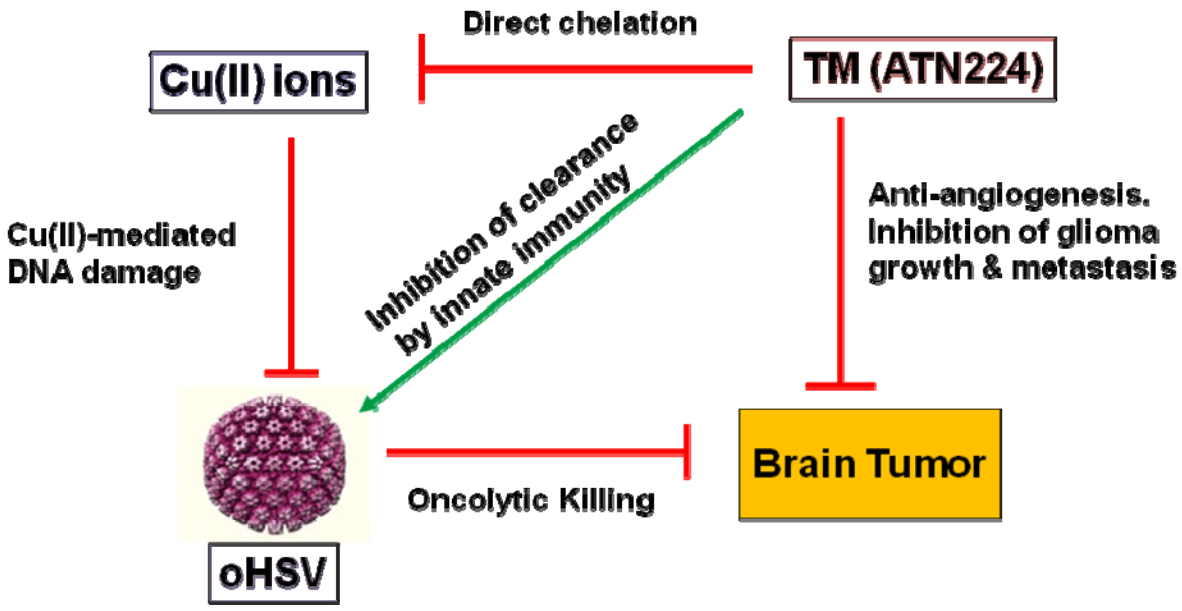


Figure 3. Proposed mechanism of ATN-224 action. We propose that ATN-224 will inhibit glioma growth and metastasis through its anti-angiogenic properties, and we expect the OV to directly kill cancer cells as well. In order to prevent copper-mediated DNA damage to OV, we propose that ATN-224 will chelate copper (II) ions to negate this effect. Finally, we hypothesize that ATN-224 will also inhibit clearance of virus by innate immunity, thus enhancing the overall anti-tumor efficacy of OV therapy.

Chapter 2: Materials and Methods

Chemicals

Cupric chloride ($CuCl_2 \bullet H_20$), ascorbic acid and Crystal Violet powder were purchased from Sigma Chemical Co. (St. Louis, MO). Tetrathiomolybdate (TM) analog, ATN-224, was kindly provided by Theodoros Teknos, MD (The Ohio State University, Columbus, OH). ATN-224 stock solution was created in phosphate buffered solution (PBS) at a concentration of 10 mM and was subsequently sterilized by passage through 0.22 um pore size membrane filters (Millex GP; Millipore, Billerica, MA). Fresh ATN-224 stock solution was made for each experiment that utilized the drug. Cupric chloride was dissolved in distilled water (dH_2O) to a concentration of 7 g/L and passed through a 0.22 um filter. Ascorbate buffer was created by dissolving ascorbic acid in HBSS at a concentration of 300 mg/L. For *in vitro* experiments, ascorbate buffer was used as a reducing agent to create more copper (II) ions in order to facilitate viral DNA damage. Serial dilutions (1:10) were prepared in ascorbate buffer to make the final Cu(II) concentration 7 mg/L. Cells in control group were treated with ascorbate buffer.

Viruses and Cell Lines

rQnestin34.5 is a second-generation oncolytic virus derived from herpes simplex virus type 1 (HSV-1) F strain, whose construction and generation has been previously described[9]. It is characterized by insertion of the green fluorescent protein (GFP) gene into the HSV-1 *UL39* locus, which inactivates the viral *ICP6* gene coding for the large subunit of ribonucleotide reductase. rQnestin34.5 also carries deletions in both copies of the *γ-34.5* gene, whose protein product is necessary for preventing the shutdown of eukaryotic protein translation by the host cell. rQnestin34.5 has one copy of the *γ-34.5*

gene reinserted under a tumor-specific synthetic nestin promoter. hrR3 is another genetically engineered HSV-1 mutant, derived from HSV-1 KOS strain, with a disruption of the *UL39* locus by insertion of the *Escherichia coli lacZ* gene under control of the *ICP6* promoter[13]. Viral stocks were generated in Vero African green monkey kidney cells (American Type Culture Collection, Manassas, VA) as previously described[40]. Human U87ΔEGFR glioma cells from our laboratory were stably transfected with a plasmid that expresses a constitutively active mutant form of epidermal growth factor receptor (EGFR), were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100-U/mL penicillin, 100-µg/mL streptomycin, and 200-µg/mL G418. Human Gli36ΔEGFR gliomas from our laboratory were also stably transfected with an EGFR plasmid and maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100-U/mL penicillin, 100-µg/mL streptomycin, 2-µg/mL puromycin and 4-µg/mL blasticidin. Human U251T3 gliomas and patient-derived X12V2 gliomas were cultured in DMEM supplemented with fetal bovine serum (10% for U251T3, 2% for X12V2), 2 mM glutamine, 100-U/mL penicillin, and 100-µg/mL streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere at 5% CO_2.

***In vitro* viral replication assay**

The day before infection, $4.5x10^5$ gliomas were seeded in DMEM 2% FBS in a 6-cm plate. The day of infection, gliomas were infected with oncolytic virus (OV) at a multiplicity of infection (MOI) of 0.1. Prior to infection, ATN-224 was incubated with copper for 1 hour at RT, and then OV was incubated with ascorbate buffer ± copper (1 mg/L) ± ATN-224 (32 uM) for 30 min at RT. Gliomas were infected with OV and

incubated at 37°C for 30 min, then cells were washed with PBS for removal of unattached virus. At 48 hours post virus infection, cells and media were collected and freeze-thawed three times to release the viruses. The number of infectious particles (viral titer) present in the resulting supernatants was then determined by performing a standard plaque forming unit assay on Vero cells, as previously described[41].

***In vitro* cytotoxicity assay**

The day before infection, 5000 glioma cells/well were seeded in DMEM 2% FBS in a 96-well plate. The day of infection, gliomas were infected with oncolytic virus (OV) at a multiplicity of infection (MOI) of 0.1. Prior to infection, ATN-224 was incubated with copper for 1 hour at RT, and then OV was incubated with ascorbate buffer ± copper (1 mg/L) ± ATN-224 (32 uM) for 30 min at RT. Gliomas were infected with OV, and cell survival was measured via crystal violet staining 48 hours post infection. Cells were fixed with 1% glutaraldehyde for 15 min at RT then stained with 100 μL/well of 0.5% Crystal Violet (5 g Crystal Violet powder + 50 mL 95% EtOH + ddH_2O = total 1000 mL solution) for 15 min while shaking. Cells were gently washed in water and allowed to dry. Crystals were dissolved in 100 μL/well of Sorenson's buffer (3.68 g sodium citrate and 12.5 mL of 0.025 M citric acid in 487.5 mL of 50% EtOH). Absorbance was measured in a plate reader at 590 nm.

Animal Studies

All mice experiments have been approved by the Institutional Review Board, and all mice were housed and handled in accordance with the guidelines of the Subcommittee on Research Animal Care of The Ohio State University. Female athymic

nude (nu/nu) mice were purchased (Charles River Laboratories, Frederick, MD) at 6-8 weeks old for all *in vivo* experiments.

For subcutaneous tumor studies, U251T3 human gliomas ($1.5x10^7$ cells) were implanted subcutaneously into the rear flank of nude mice. When tumors reached an average size of 100 mm^3, tumor-implanted mice were randomly assigned and treated daily with ATN-224 (0.7 mg/mouse dissolved in 100 uL PBS) or PBS (100 uL) by gavage technique (n=10). Gavage is a method of force-feeding that involves plunging liquid down the mouse's esophagus, so as to ensure equivalent uptake of the drug by all mice. At day 13 from the initial ATN-224 treatment, rQnestin34.5 ($5x10^5$ pfu) or PBS was then administered by a single intratumoral injection. Tumor size was measured every day, and tumor volume was calculated using the following formula: volume = $0.5LW^2$.

For intracranial tumor studies, anesthetized nude mice were fixed in a stereotactic apparatus, and a burr hole was drilled at 2 mm lateral to the bregma, to a depth of 3 mm. U87ΔEGFR human gliomas ($1x10^5$ cells in 4 µl Hank's buffered salt solution [HBSS]) were implanted. On day 3 after tumor implantation, mice were randomly assigned and treated by daily gavage with ATN-224 (0.7 mg/mouse dissolved in 100 uL PBS) or PBS (100 uL). On day 10 after tumor implantation, mice were anesthetized again and stereotactically inoculated with a single intratumoral injection of rQnestin34.5 ($5x10^4$ pfu in 4 uL HBSS) or an equivalent volume (4 uL) of PBS at the same location as the tumor implantation. Animals were observed daily and were euthanized at the indicated time points or when they showed signs of morbidity, i.e. when mice become moribund, lethargic, anorexic, dehydrated or distressed.

Histology and Immunohistochemistry

Tumors were fixed in 4% buffered paraformaldehyde followed by 30% sucrose at 4°C, then embedded in OCT compound (Tissue Tek, Sakura Finetec, Torrance, CA) and cut into 10-μm thick sections. Representative sections were stained with hematoxylin & eosin (H&E) and examined by light microscopy. To observe microvessel density (MVD), cryosections were treated with purified mouse anti-CD31 (Pharmingen, San Jose, CA) as a primary antibody to visualize endothelial cells lining the blood vessels (n=3 mice/group), were treated with biotin-SP-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as a secondary antibody, and then were incubated with Vectastain ABC reagent (Vectastain ABC kit, Vector Labs, Burlingame, CA) for 30 min at RT. Diaminobenzidine/hydrogen peroxidase (Thermo Scientific, Rockford, IL) was used as the chromogen substrate. All slides were counterstained with Meyer's hematoxylin. The three most vascularized areas within the tumor ("hot spots") were chosen at low magnification, and vessels were counted in a representative high magnification (10x) field in each view field[42]. Single immunoreactive endothelial cells or endothelial cell clusters separate from other microvessels were counted as individual microvessels. Mean MVD was calculated as the average number of counts per view field. Vessels at the periphery of the tumor were disregarded in the MVD counts. The MVD for each therapy group was then averaged together [n=18 view fields/group (3 view fields/section and 2 sections/tumor and 3 tumors/group)] to obtain the final count ± SEM.

Analysis of Serum Inhibition of OV

Mice were divided into two groups and treated by daily gavage with ATN-224 (0.7 mg/mouse dissolved in 100 uL PBS) or PBS (100 uL) (n=6). On day 7 after the initial ATN-224 treatment, hrR3 ($2x10^7$ pfu) was administered intravenously by a tail vein injection. Blood was then drawn from the sub-mandibular vein 20 minutes after hrR3 injection, blood was spun down for 20 min at 6000 rpm, and virus-containing serum supernatant was collected. Mouse serum (5 uL) was incubated with HBSS (45 uL) briefly before infecting Vero cells in 48-well plates**.** Virus titer was determined by viral plaque assay[41]. DMEM media with 2% fetal bovine serum was added 1 hour later, and cells were cultured overnight. At 16 hours post infection, cells were treated with 4% human IgG, and plaque number was counted 3 days after infection. Plaque numbers corresponding to each virus in serum were then converted to virus titer using a standard curve obtained for rQnestin34.5 (at multiple known titers) pre-incubated with HBSS.

***In vivo* Tumoral Detection of OV**

U251T3 human gliomas ($1.5x10^7$ cells) were implanted subcutaneously into the rear flank of nude mice. When tumor volumes reached approximately 250 mm^3, tumor-implanted mice were randomly assigned and treated by daily gavage with ATN-224 (0.7 mg/mouse dissolved in 100 uL PBS) or PBS (100 uL) for 7 days.

When analyzing intratumoral administration of OV, rQnestin34.5 ($5x10^5$ pfu) was injected intratumorally on day 7 and on day 9 after the initial ATN-224 (or PBS) treatment, for a total of two injections (n=8). On day 12, three days after the final OV

injection, tumors were harvested and processed for virus titration. Briefly, tumors were collected and minced into small pieces before incubation for 1 min at 37°C with the following enzymes dissolved in HBSS: collagenase Type I (0.05 mg/ml), hyaluronidase (0.025 mg/ml), and soybean trypsin inhibitor (0.2 trypsin inhibitor unit/ml, Roche Diagnostics, Nutley, NJ). The lysed tumors were centrifuged at 8000 rpm for 20 min, and supernatant was stored -80°C. Virus titers were determined by plaque forming unit assay in Vero cells[41]. *In vivo* replication results were normalized relative to the total tumor weight in each sample (by lysing tumor cells using an adjusted volume of lysis buffer) and were calculated as pfu/mL of lysis buffer.

When evaluating intravenous administration of OV, hrR3 ($1x10^7$ pfu) was injected intravenously by a tail vein injection on day 7 after the initial ATN-224 (or PBS) treatment, one time (n=3). On day 10, subcutaneous tumors were harvested, and total DNA was purified using the MasterPure™ Complete DNA & RNA Purification Kit (Cat. No. MC85200, Epicentre Biotechnologies), according to the manufacturer's instructions. OV presence was measured by determining the total number of copies of the HSV-specific *ICP4* gene ("OV gene copy") present in the tumors using absolute quantitative real-time PCR (qPCR) analysis. Absolute qPCR was performed using ABI 7500 Standard Block machine (Applied Biosciences), and each sample was performed in triplicate. The following *ICP4* primer was used: sense, 5'-CGACACGGATCCACGACCC-3' and antisense, 5'-GATCCCCCTCCCGCGCTTCGTCCG-3'. Total OV gene copy was determined by generating a linear regression curve using a pK1-2 plasmid containing the *ICP4* HSV-1 viral gene, kindly provided by Dr. Deborah Parris at The Ohio State University. The

plasmid was diluted to various concentrations ranging from 100 ng/5 uL to 0.0005 ng/5 uL DNA. The number of plasmids per ug of DNA was calculated using the total number of nucleotides in the plasmid [(amount(ng)*6.022x10^{23})/(length*1x10^{9}*660)]. qPCR was performed with the diluted concentrations of the plasmid, and CT value versus gene copy number was plotted to give the linear regression standard curve. qPCR samples (20 uL per well) were loaded into a 1% agarose gel, and ICP4 gene amplification was confirmed by agarose gel electrophoresis post-qPCR.

Statistical Analysis

Student's t-test was used to analyze data for viral replication, glioma cell survival in cytotoxicity assay, microvessel density and gene copy changes. Two-tailed P-values are displayed. A P-value <0.05 was considered statistically significant in student's t-test. All error bars displayed are shown as standard deviations, and all graphs were generated with use of Microsoft Excel. To analyze mouse survival data, Kaplan-Meier curves were compared using the log rank and Breslow (Generalized Wilcoxon) tests. All statistical analyses were performed with the use of SPSS statistical software (version 14.0; SPSS, Chicago, IL).

Chapter 3: Results

ATN-224 Rescues Copper Inhibition of OV *in vitro*

Copper has been shown to inhibit wild-type HSV-1 through a DNA-damaging mechanism induced by copper (II) ions in a reducing environment[32]. Through its ability to chelate copper (II) ions, ATN-224 has the potential to remove copper and its inhibitory effects against oncolytic virus (OV) therapy from the tumor microenvironment. To test whether ATN-224 can rescue copper inhibition of oncolytic HSV-1 (oHSV), we infected gliomas with rQnestin34.5 (for simplicity, referred to as OV) that had just previously been incubated with ascorbate buffer (OV alone), buffer and copper (OV+copper), or buffer and copper and ATN-224 (OV+copper+ATN224). We quantified the results as a measure of total amount of infectious viral particles (for viral replication) or as a measure of the percentage of glioma cells that survived treatment (for cytotoxicity).

We first observed that copper, at a physiological concentration of 1 mg/L in ascorbate buffer (300 mg/L in HBSS), markedly reduced the ability of rQnestin34.5 oncolytic virus to replicate *in vitro* in the human glioma cells U251T3 and Gli36ΔEGFR and the patient-derived glioma cells X12V2. When gliomas ($4.5x10^5$ cells/plate) were treated with virus that had been previously incubated with copper and ATN-224, we demonstrated that ATN-224 can restore the ability of rQnestin34.5 to replicate, as evidenced by a greater viral load in U251T3 gliomas (total plaque-forming units [pfu] following infection, OV+copper vs. OV+copper+ATN224: $1.5625x10^0$ vs. 3.0469×10^5, P=0.001052), in X12V2 gliomas (total pfu following infection, OV+copper vs. OV+copper+ATN224: $7.8125x10^{-1}$ vs. $2.8125x10^5$, P=0.0001053), and in Gli36ΔEGFR

gliomas (total pfu following infection, OV+copper vs. OV+copper+ATN224: $0.00x10^0$ vs. $3.3875x10^4$, P=0.0005461) (Figure 4A).

Similarly, copper (1 mg/L) significantly reduced the cytotoxicity of rQnestin34.5 in human glioma lines U251T3 and Gli36ΔEGFR and in patient-derived glioma cells X12V2. When gliomas (5000 cells/well) were treated with virus that had been previously incubated with copper and ATN-224, we demonstrated that ATN-224 can restore the ability of rQnestin34.5 to kill gliomas, as evidenced by reduced percentages of U251T3 gliomas (cell survival following infection, OV+copper vs. OV+copper+ATN224, compared to untreated control: 78.0% vs. 25.7%, P<0.0001), X12V2 gliomas (cell survival following infection, OV+copper vs. OV+copper+ATN224, compared to untreated control: 73.4% vs. 20.9%, P<0.0001), and Gli36ΔEGFR gliomas (cell survival following infection, OV+copper vs. OV+copper+ATN224, compared to untreated control: 97.1% vs. 18.7%, P<0.0001) that survived treatment (Figure 4B).

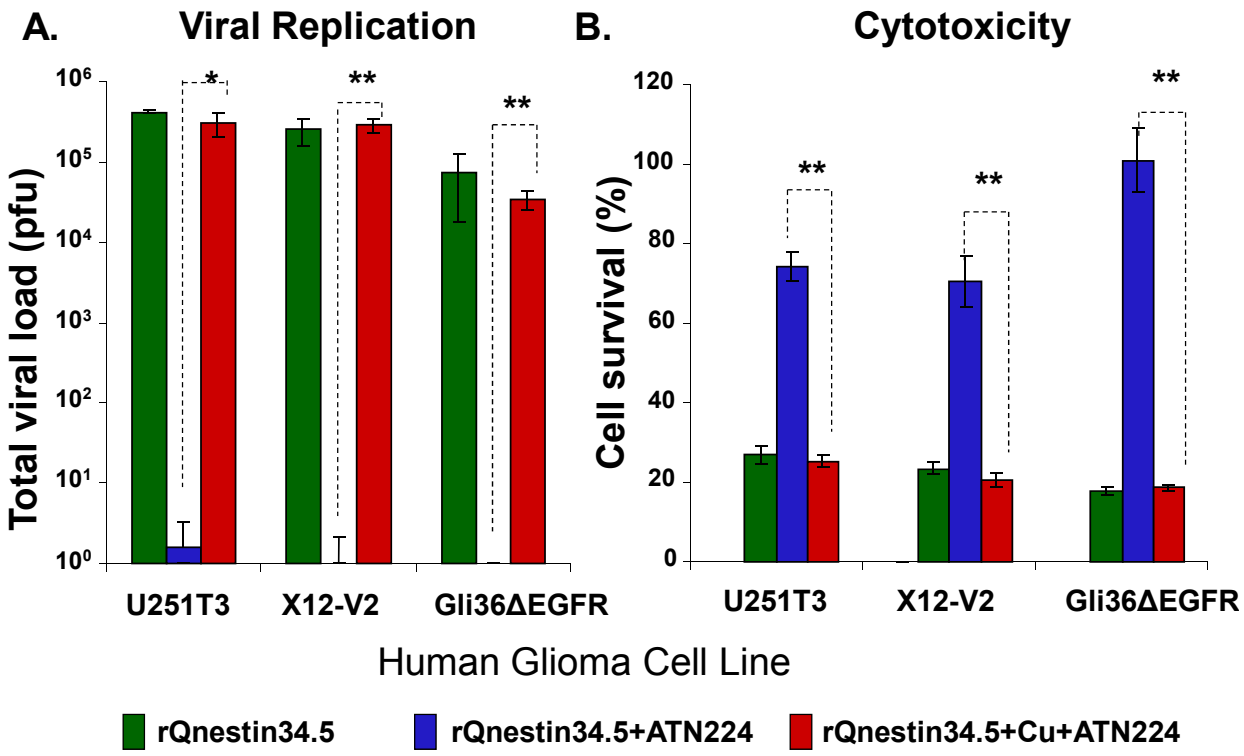


Figure 4. ATN-224 rescues copper inhibition of OV replication and OV-mediated glioma cell killing *in vitro*. Gliomas (U251T3, X12V2 and Gli36ΔEGFR) were infected with rQnestin34.5 oncolytic virus (OV) at multiplicity of infection (MOI) of 0.1. Prior to infection, ATN-224 was incubated with copper, and then OV was incubated with ascorbate buffer ± copper (1 mg/L) ± ATN-224 (32 uM). **A)** Gliomas ($4.5x10^5$ cells/plate) were infected with rQnestin34.5, and cells were harvested for viral isolation 48 hours post infection. Viral titers were measured via fluorescence microscopy quantification of green fluorescent protein (GFP) expression by rQnestin34.5-infected cells. Total viral load was increased in cells infected with OV+copper+ATN224 compared to cells infected with OV+copper, and was comparable to cells infected with OV alone. **B)** Gliomas (5000 cells/well) were infected with rQnestin34.5, and cell survival was measured via crystal violet staining 48 hours post infection. Glioma survival was decreased in cells infected with OV+copper+ATN224 compared to cells infected with OV+copper, and was comparable to cells infected with OV alone (**P<0.001, *P<0.01).

In the Absence of Copper, ATN-224 Has No Effect on OV Cytotoxicity *in vitro*

To our knowledge, ATN-224 has never been tested for its effects on modulating the efficacy of any oncolytic virus. As a safety checkpoint, we searched for any potential antagonistic effects that ATN-224 could have against rQnestin34.5 oncolytic virus. Determining if ATN-224 acts by some mechanism to disrupt the efficacy of our original OV therapy would indicate whether ATN-224 administration would need to precede OV treatment (if found to be antagonistic) or whether ATN-224 could be administered concurrently with OV (if found to display no effect) in future *in vivo* studies.

To test this, glioma cells (U251T3, X12V2 and Gli36ΔEGFR, 5000 cells/well) were plated in a 96-well plate and were infected with OV (rQnestin34.5) at a multiplicity of infection (MOI) of 0.1. Prior to infection, OV was incubated with or without ATN-224 (32 uM) to assess the effect of ATN-224 on a constant amount of OV. Cytotoxicity was assessed as a measure of percentage of cells surviving infection, compared to untreated control, and was quantified by crystal violet staining 48 hours post infection. Results in all three glioma cell lines show that, in the absence of copper, ATN-224 has no significant effect on the ability of rQnestin34.5 to kill glioma cells (Figure 5).

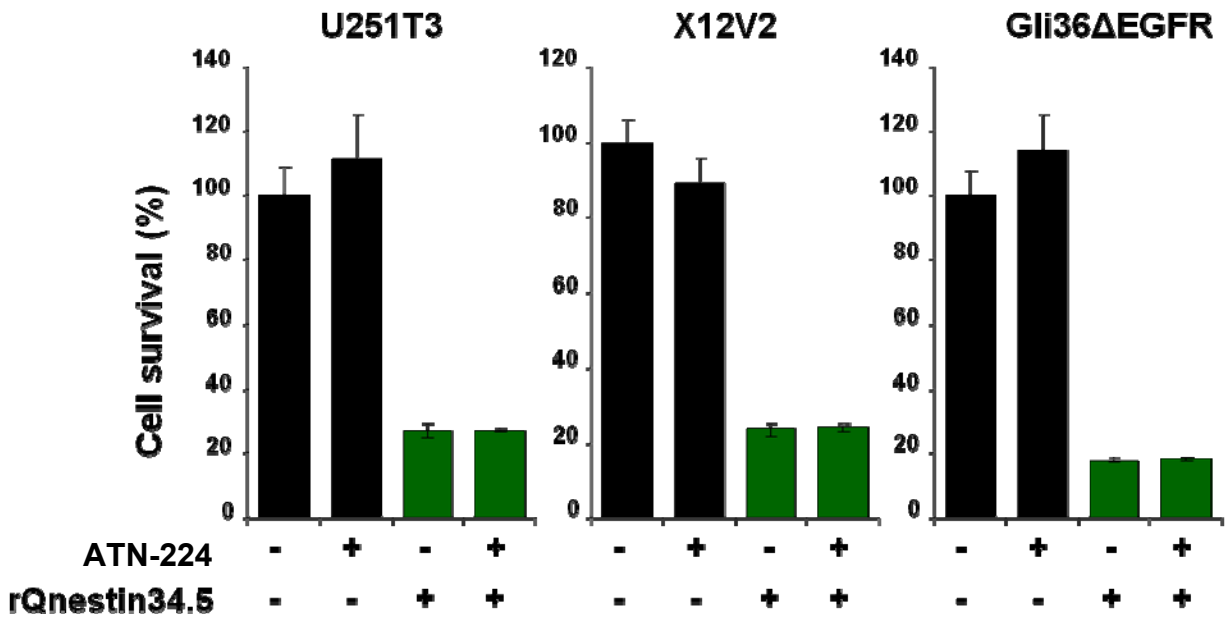


Figure 5. In the absence of copper, ATN-224 does not affect OV-mediated cytotoxic killing in vitro. Glioma cells (U251T3, X12V2 and Gli36ΔEGFR) were infected with OV (rQnestin34.5), ATN-224, or both OV+ATN224. Cell survival was measured via crystal violet staining 48 hours post infection. ATN-224 (32 uM) showed no significant effect on glioma cell growth following OV infection.

ATN-224 Enhances Anti-Tumor Efficacy of OV Therapy *in vivo*

Having demonstrated the ability of ATN-224 to significantly rescue the inhibitory effects of copper on OV replication and cytotoxicity *in vitro* without by itself adversely affecting OV therapy, we were encouraged to test our hypothesis *in vivo*. This study implemented two *in vivo* models to assess the effect of ATN-224 on the anti-tumor efficacy of OV therapy. To do this, we measured tumor size in a subcutaneous xenograft glioma model and evaluated mouse survival in an intracranial xenograft glioma model. In all of the *in vivo* experiments in this project, ATN-224 (0.7 mg/mouse dissolved in 100 uL PBS) was systemically administered daily via gavage technique, as previously described[26].

To study tumor size, U251T3 human gliomas ($1.5x10^7$ cells) were implanted subcutaneously into athymic nude mice and were treated with PBS, ATN-224, OV, or OV+ATN224 (n=10). The oncolytic virus utilized in this study was rQnestin34.5. When tumor sizes reached approximately 100 mm^3, daily ATN-224 or PBS treatment began. On day 13 after the initial ATN-224 treatment, tumors were treated with a single intratumoral injection of OV (rQnestin34.5, $5x10^5$ pfu) or PBS. Tumor volumes were measured every other day throughout the experiment. Our results indicate that 25 days after the initial ATN-224 treatment, tumors treated with a combination of ATN-224 with rQnestin34.5 displayed significantly smaller volumes compared to tumors treated with rQnestin34.5 alone (21.51 vs. 153.93 mm^3, P=0.0383) (Figure 6A). A complete response is indicated by the complete regression of a tumor; that is, an impalpable tumor. In each of the ATN-224 alone and the OV alone experimental groups, 1 of the 10 mice made a complete response. However, of the mice treated with a combination of

ATN-224 with OV, 7 of the 10 mice exhibited a complete response. After noting the tumor-regressing effect of ATN-224 and OV combination therapy in subcutaneous tumors, we were interested to see if this anti-tumor effect would also be observed in an intracranial tumor model assessing mouse survival.

To test this, U87ΔEGFR human gliomas ($1x10^5$ cells) were implanted intracranially in athymic nude mice and were treated with PBS, ATN-224, OV, or OV+ATN224. Intracranial surgery was performed for mice in the PBS and ATN-224 treatment groups on one day (n=8) and for mice in the OV and OV+ATN224 treatment groups on the next day (n=10) in order to distribute the workload of a large surgery. Mice from both surgery days were treated under the exact same experimental conditions and with the exact same treatment regimens. On day 3 after tumor implantation, daily ATN-224 or PBS treatment began. On day 10 after tumor implantation (after 7 days of ATN-224 treatment), intracranial tumors were treated with a single intratumoral injection of OV (rQnestin34.5, $5x10^4$ pfu) or PBS. Mice were monitored closely for signs of morbidity, at which point mice were killed by cervical dislocation. Kaplan-Meier analysis revealed a statistically significant increase in mean survivals for mice treated with combined ATN-224 and rQnestin34.5 compared to mice treated with rQnestin34.5 alone (32.000 vs. 18.600 days, Breslow [generalized Wilcoxon] significance test score=0.044) (Figure 6B).

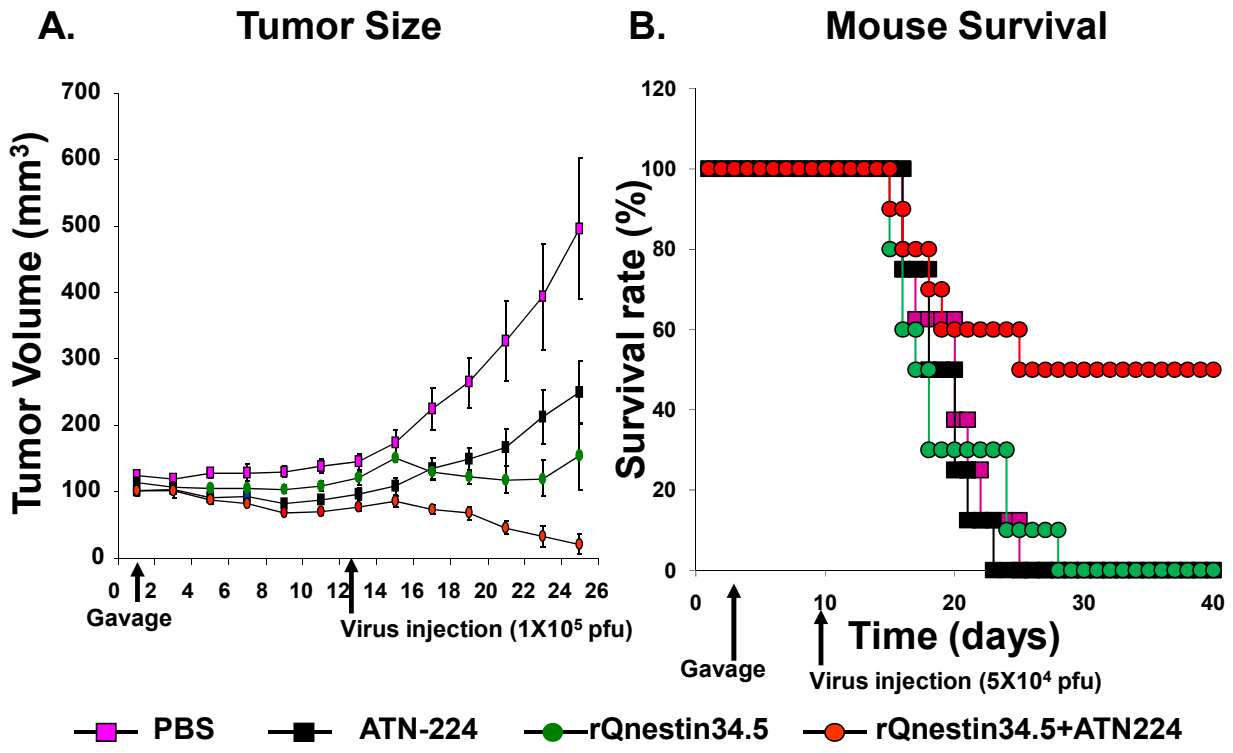


Figure 6. ATN-224 enhances anti-tumor efficacy of OV therapy in vivo. ATN-224 or PBS was systemically administered daily by gavage. **A)** U251T3 glioma cells ($1.5x10^7$ cells) were implanted subcutaneously in athymic nude mice, and mice were treated with PBS, ATN-224, OV, or OV+ATN224 (n=10). OV (rQnestin34.5, $5x10^5$ pfu) was injected intratumorally on day 13 after initial ATN-224 treatment. Tumor size was measured every other day. Mice treated with combined ATN-224 and OV displayed reduced tumor sizes compared to mice treated with OV alone (P=0.0383). **B)** Kaplan-Meier survival curve of mice implanted with intracranial U87ΔEGFR gliomas ($1x10^5$ cells) and treated with PBS, ATN-224, OV, or OV+ATN224 (n=8). OV (rQnestin34.5, $5x10^4$ pfu) was injected intratumorally 7 days after initial ATN-224 treatment. Mouse survival was greater for mice treated with combined ATN-224 and OV compared to mice treated with OV alone (Breslow significance test score=0.044).

These results suggest that ATN-224 represents a promising co-therapeutic agent that can enhance the anti-tumor efficacy of OV therapy *in vivo*. We next sought to identify the physiological mechanisms through which ATN-224 manifests this augmentation of OV therapy.

ATN-224 Decreases Angiogenesis *in vivo*

To begin to answer the question as to how specifically ATN-224 is able to enhance the efficacy of OV therapy, we decided to examine the effect of ATN-224 on tumor angiogenesis following OV treatment. Kurozumi et al. used an anti-angiogenic drug to normalize tumor vasculature, resulting in increased titers of oncolytic virus and extended mouse survival[20]. Therefore, we wanted to test if the improved efficacy of OV therapy we observed *in vivo* could be attributed to the ability of ATN-224 to reduce the number of blood vessels present in the tumor microenvironment.

Here, U251T3 human gliomas ($1.5x10^7$ cells) were implanted subcutaneously into athymic nude mice and were treated with PBS, ATN-224, OV, or OV+ATN224 (n=3). When tumor sizes reached approximately 250 mm^3, daily ATN-224 or PBS treatment began. On day 7 after initial ATN-224 treatment, tumors were treated with a single intratumoral injection of OV (rQnestin34.5, $5x10^5$ pfu) or PBS. On day 10, subcutaneous tumors were harvested, sectioned and stained for CD31, which detects endothelial cells that line blood vessels[20]. Blood vessel quantification was determined by counting the number of CD31-immunoreactive endothelial cell clusters as individual microvessels and calculating an average count per view field. Tumors treated with ATN-224 had a significantly lower microvessel density than did tumors treated with PBS control (ATN-224 vs. PBS: 7.89 vs. 18.72 CD31-positive microvessels/view field,

P<0.001; OV+ATN224 vs. OV: 6.72 vs. 16.56 CD31-positive microvessels/view field, P<0.001), confirming the anti-angiogenic property of ATN-224 (Figure 7). No significant difference was observed in microvessel density between tumors treated with ATN-224 and OV+ATN224.

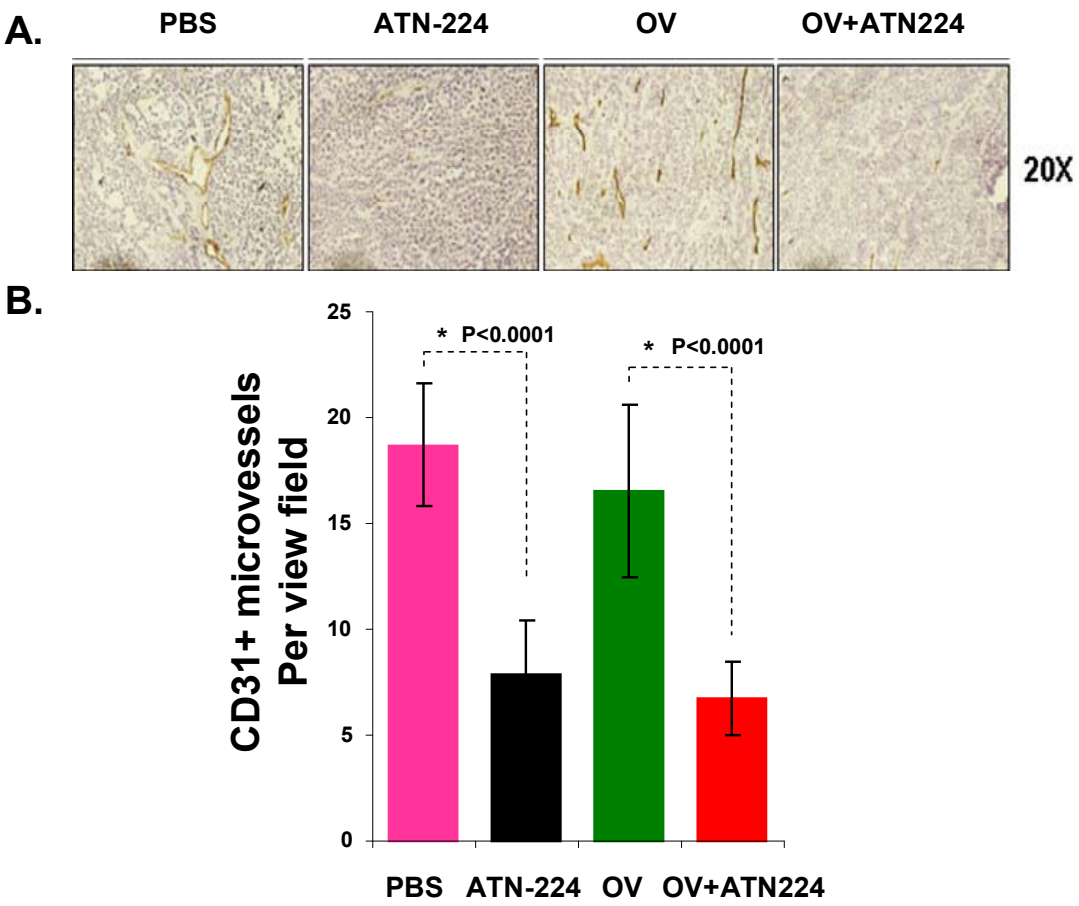


Figure 7. Reduced vasculature detected in subcutaneous tumors treated with ATN-224. U251T3 gliomas ($1.5x10^7$ cells) were implanted subcutaneously in athymic nude mice. Mice were treated with ATN-224 or PBS via gavage every day (n=3). On day 7 after initial ATN-224 treatment, OV (rQnestin34.5, $5x10^5$ pfu) was directly injected into the tumor. On day 10, tumors were harvested. A) Immunohistochemistry, CD31 stain (red) on subcutaneous tumor sections, 20x magnification. B) Quantification of CD31-immunoreactive microvessels, 10x magnification. Tumors treated with ATN-224 had lower microvessel density than did tumors treated with PBS.

ATN-224 Rescues OV from Serum Inhibition *in vivo*

Although host defense mechanisms have been recognized to limit oncolytic virus propagation[22, 43-44], much fewer studies have examined the effect of angiogenesis—and anti-angiogenic drugs, in particular—on OV replication[20]. We suspected that the anti-angiogenic property of ATN-224 observed above only partly contributed to the enhanced efficacy of OV therapy *in vivo*. Because ATN-224 chelates copper and, theoretically, prevents Cu(II)-mediated DNA damage to our oncolytic HSV, perhaps the improved anti-tumor efficacy of OV therapy *in vivo* was also due to increased levels of virus available for glioma destruction following ATN-224 treatment.

Our first step in assessing the effect of ATN-224 on OV replication was to examine the amount of virus detected in the serum of mice treated with or without ATN-224. Mice were divided into two groups and treated daily with ATN-224 or PBS (n=6). On day 7 after the initial ATN-224 treatment, OV (hrR3, $2x10^7$ pfu) was administered intravenously by a tail vein injection. In this experiment, we chose to administer hrR3 oncolytic virus instead of rQnestin34.5 (as we had previously used in this study) because hrR3 had been shown to persist in mouse serum in one study[13] and had been delivered intravenously in other studies[10, 45], whereas rQnestin34.5 had not been tested in this manner before (i.e. intravenous administration). Therefore, we believed that hrR3 would be the more appropriate OV to test using an intravenous administration of virus. Blood was drawn from the sub-mandibular vein 20 minutes after OV injection, and virus titer was determined by viral plaque assay. Results indicate that mouse serum treated with OV+ATN224 permitted the survival of more virus than did mouse serum treated with OV alone ($1.85x10^5$ vs. $5.25x10^4$ pfu/mL, P=0.004) (Figure 8).

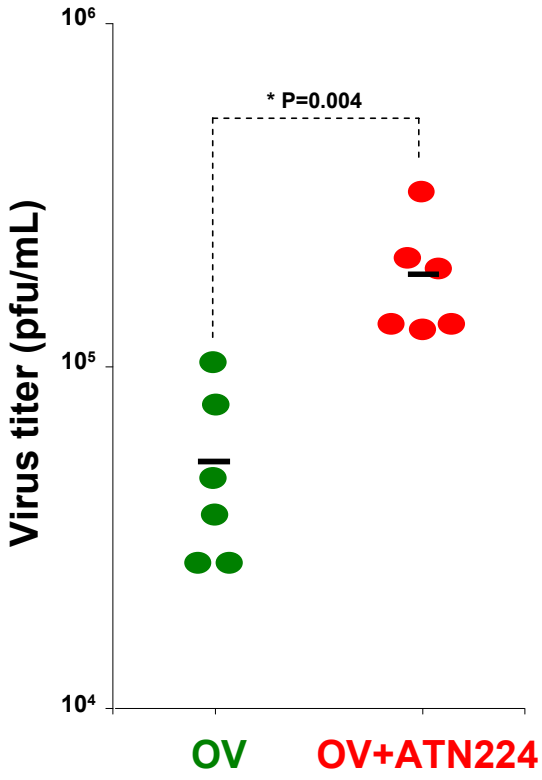


Figure 8. Increased viral presence in mouse serum treated with ATN-224. Mice were treated with ATN-224 or PBS via daily gavage (n=6). On day 7 after initial ATN-224 treatment, OV (hrR3, $2x10^7$ pfu) was administered intravenously. Twenty minutes after OV injection, venous blood was collected, and virus titer was determined via viral plaque assay. Plaque numbers corresponding to each virus in serum sample were converted to virus titer using standard curve obtained for hrR3 virus pre-incubated with HBSS. Mouse serum treated with ATN-224 permitted survival of more virus than did mouse serum treated with PBS (P=0.004).

ATN-224 Increases OV Presence in Tumors *in vivo*

To make a more clinically relevant assessment of the effect of ATN-224 on OV propagation *in vivo*, we next examined the amount of virus detected within the actual tumors of mice that had or had not undergone ATN-224 treatment. In both of the following experiments, U251T3 human gliomas ($1.5x10^7$ cells) were implanted subcutaneously into athymic nude mice, and mice were treated daily with either ATN-224 or PBS when tumor sizes reached approximately 250 mm^3.

In the first experiment, OV (rQnestin34.5, $5x10^5$ pfu) was injected intratumorally on day 7 and on day 9 after the initial ATN-224 (or PBS) treatment, for a total of two injections (n=8). On day 12, three days after the final OV injection, tumors were harvested and processed for virus titration, quantified by a viral plaque assay. With the intratumoral administration of rQnestin34.5, subcutaneous tumors that were treated with OV+ATN224 yielded higher viral titers than did tumors treated with OV alone ($3.71x10^4$ vs. $1.12x10^4$ pfu/mL, P=0.018) (Figure 9A).

In the second experiment, OV (hrR3, $1x10^7$ pfu) was injected intravenously by a tail vein injection on day 7 after the initial ATN-224 (or PBS) treatment, one time (n=5). As before, our reasoning for utilizing hrR3 oncolytic virus instead of rQnestin34.5 was due to the previously demonstrated intravenous use of hrR3 in other studies[10, 45], and lack of this literature for rQnestin34.5. On day 10, subcutaneous tumors were harvested, and total DNA was purified. OV presence was measured by determining the number of copies of the HSV-specific ICP4 gene ("OV gene") present in the tumors using quantitative real-time PCR (qPCR) analysis, confirmed by agarose gel electrophoresis post-qPCR (Figure 9B, 9C). Here, with the intravenous administration of

hrR3, tumors treated with OV+ATN224 also revealed increased presence of virus compared to tumors treated with OV alone ($2.81x10^5$ vs. $1.27x10^5$ copies of ICP4 viral gene, P=0.0491).

These results suggest that the enhanced *in vivo* efficacy of OV therapy against malignant glioma may be attributed to the increased levels of virus present within the tumor and the tumor microenvironment as a result of ATN-224 treatment.

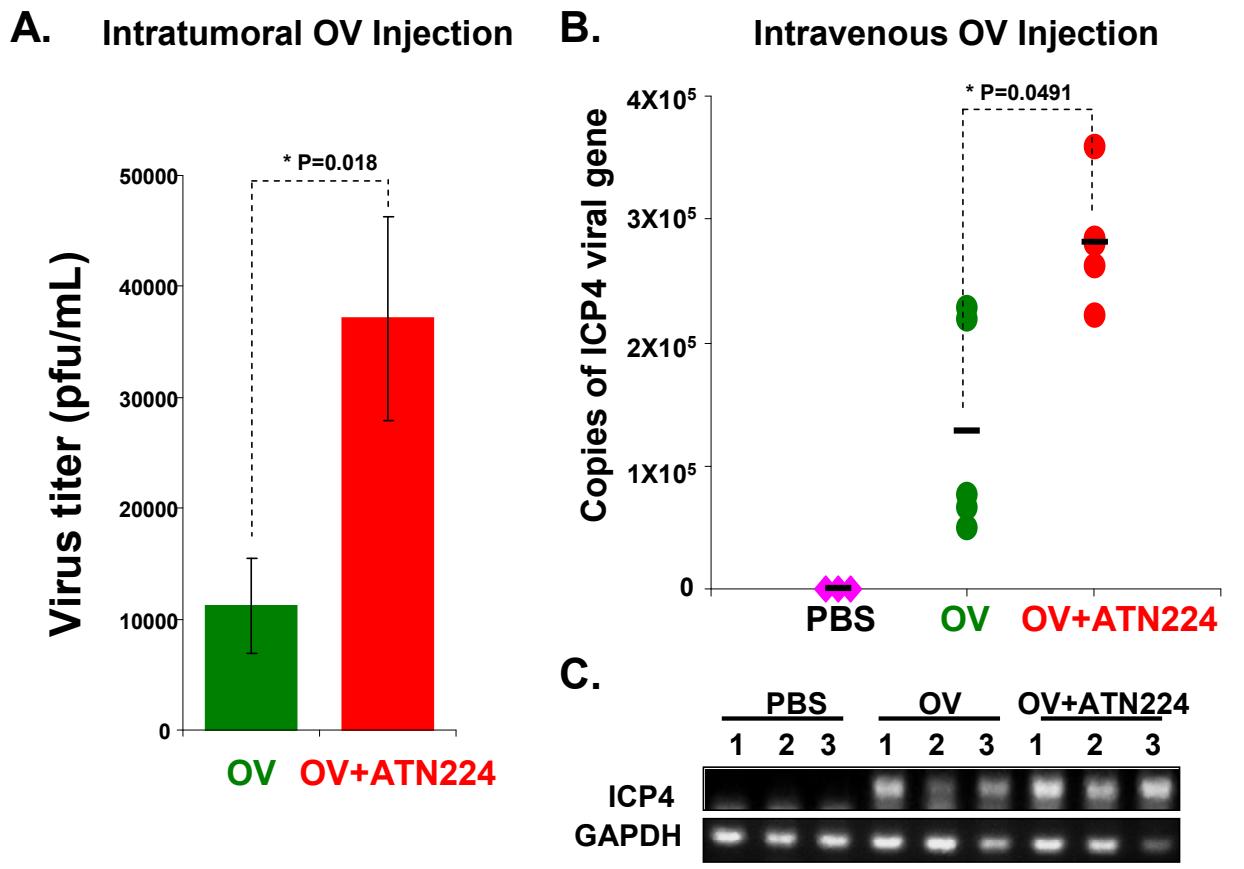


Figure 9. Increased virus detection in subcutaneous tumors treated with OV+ATN224 compared to OV alone *in vivo*. U251T3 glioma xenografts ($1.5x10^7$ cells) were established subcutaneously in nude mice. Mice were treated daily with either ATN-224 or PBS, administered systemically by gavage. **A)** On day 7 after initial ATN-224 treatment, OV (rQnestin34.5, $5×10^5$ pfu) was injected intratumorally, and again on day 9, for a total of two injections. Three days after the final injection of rQnestin34.5 virus, tumors were harvested and processed for virus titration, quantified by viral plaque assay (n=8). **B)** On day 7 after ATN-224 treatment began, OV (hrR3, $1x10^7$ pfu) was injected intravenously. On day 10, tumors were harvested and total DNA was purified. *ICP4* gene copy (HSV-specific) was quantified by qPCR analysis. **C)** Using DNA from qPCR samples of tumors treated with intravenous OV, confirmation of *ICP4* gene via agarose gel electrophoresis. Data shows increased viral presence in tumors treated with OV+ATN224 compared to tumors treated with OV alone.

Discussion and Conclusions

In clinical trials, oncolytic HSV (oHSV) therapy has demonstrated a remarkable tolerance, with no evidence of dose-related toxicity in patients. Evidence has shown that oHSV replication has been limited to tumor cells and has not been associated with any reactivation of latent wild-type HSV-1[46]. However, the results of these studies have been inconclusive with respect to the effect of oncolytic virus therapy on patient survival. Angiogenesis is critical for invasive tumor growth and plays an important role in tumor progression and its response to therapy[47]. It has been shown that copper plays an essential role in angiogenesis, acting as a critical co-factor for several angiogenic growth factors[33]. Additionally, copper can inhibit HSV-1 replication and persistence through a DNA-damaging mechanism induced by copper (II) ions in a reducing environment[32]. It has been suggested that copper levels needed for cellular functions are much lower than copper levels needed for tumor angiogenesis[33]. Therefore, a logical approach to reducing the detrimental effects of copper on oncolytic HSV-1 therapy would be to remove copper from the tumor microenvironment. We proposed that ATN-224—a second generation analog of tetrathiomolybdate (TM), a copper-chelating agent that is currently FDA-approved for the treatment of Wilson's disease—could inhibit the formation of blood vessels in the tumor microenvironment while simultaneously preventing DNA damage to oncolytic HSV-1 by physiologic levels of copper. We hypothesized that the combination therapy of ATN-224 with oncolytic virus would reduce tumor angiogenesis and enhance the ability of oncolytic virus to destroy malignant glioma.

In this study, we demonstrated that copper, at a physiologically relevant concentration, inhibits OV replication and OV-mediated glioma killing. We showed *in vitro* that, through its chelation of copper, ATN-224 can remove the inhibitory effects of copper on OV replication and cytotoxicity, and we showed that ATN-224 by itself has no antagonistic effect on the cytotoxic ability of our original OV therapy. Our *in vivo* results indicated that mice treated with the combination of ATN-224 and OV displayed significantly reduced tumor sizes and increased mouse survival compared to mice treated with OV alone, thus demonstrating that ATN-224 treatment can enhance the anti-tumor efficacy of OV therapy *in vivo*. ATN-224 treatment was shown to reduce tumor vasculature following OV infection and to increase the presence of OV in mouse serum and in subcutaneous tumors treated both intratumorally and intravenously. Overall, these results strongly suggest that the co-treatment of ATN-224 with oncolytic virus can significantly improve the poor efficacy profile of conventional clinical oncolytic virotherapy.

The host immune response has been recognized to have an important role in limiting viral oncolysis[20, 22, 48], and the vascular endothelium is known to be an important component of host inflammatory responses[49]. The key role played by blood vessels in increasing tissue inflammation is supported by literature stating that increased microvessel density in transgenic mice engineered to overexpress vascular endothelial growth factor A (VEGF-A, a stimulator of angiogenesis) led to a significantly increased and prolonged inflammatory response compared to wild-type mice. Additionally, this amplified inflammatory response could be hindered by antibodies against the VEGF-A receptor[50].

While multiple studies have attempted to exploit anti-angiogenic treatment to control the growth of glioblastoma[51-53], few have investigated the effect of inhibiting angiogenesis in combination with OV therapy. One study in particular employed the intracranial, direct intratumoral injection of cRGD peptide (cilengitide, an angiogenesis inhibitor) as a pre-treatment to OV therapy[20]. Because this treatment regimen required two intratumoral injections (drug and virus treatment) at separate time points, translation of this dose-administration schedule to the clinics for patients with brain tumors would not be practical, as a repeat craniotomy would be cost-inefficient and risky for both the patient and the health care provider. By using ATN-224, we were able to administer anti-angiogenic treatment systemically, thus eliminating the need to directly access the tumor at multiple time points. Systemic administration of anti-angiogenic ATN-224 treatment is much more feasible in its transition to application in a clinical setting.

As the first generation analog of ATN-224, ammonium tetrathiomolybdate (TM), is currently an FDA-approved drug that is used for the treatment of Wilson's disease, the dose-response curve for this drug family is already characterized and understood for humans. The anti-angiogenic effects of TM and ATN-224 are being investigated in clinical trials as an anti-cancer therapy for multiple tumor modalities, including breast cancer, prostate cancer, and lung cancer[14]. Oncolytic viruses, such as the HSV-1-derived HSV1716 and G207 viruses, have been studied in clinical trials for their efficacy against malignant glioma[54]. The existing track record of these two treatments in human trials greatly encourages the testing of their combination, ATN-224 with oncolytic virus, as therapy for brain tumors in humans as well.

However, this study has several limitations. The data demonstrating that ATN-224 rescues the inhibitory effects of copper on OV are only displayed for three brain tumor cell types. While the general term is “brain tumor,” each cell line of brain tumors is characterized by unique behavior both in its own growth and in its response to treatment. Also, our rQnestin34.5 oncolytic virus is designed to be most effective in cell types that overexpress the nestin protein gene. To demonstrate breadth of application for ATN-224 and rQnestin34.5 combination therapy, we would need to expand our testing to include a variety of additional glioma cell lines with varying nestin expression levels. Similarly, rQnestin34.5 is an oncolytic virus that is specifically designed for therapy against malignant glioma. Collaboration with other cancer labs would be required to test the effect of ATN-224 as a co-therapeutic drug with oncolytic viruses targeted for other tumor modalities.

The dosage of ATN-224 used in this experiment was shown in a previous study that used TM to reduce copper levels to 20% of baseline[26], although the side effects of reduced copper levels were not studied in this *in vivo* investigation of combining anti-angiogenic treatment with OV therapy. Copper is also known to play a key role in heme synthesis, and anemia is the first clinical sign of copper deficiency[55]. Future *in vivo* studies could incorporate the testing of blood for copper levels as well as hematocrit in order to address the potential side effect of anemia induced by copper reduction.

The results from our *in vitro* and *in vivo* studies indicate a potential for increased viral replication following ATN-224 treatment in live models. Higher viral titers isolated from tumors from mice treated with ATN-224 and OV could potentially lead to toxic effects that are associated with an increased viral burden. Since rats and mice are

resistant to HSV infection, it would be necessary to evaluate toxicity studies in primate models.

Finally, an essential aspect that must be considered is the blood brain barrier. As the anti-angiogenic/oncolytic virus therapeutic strategy of this study targets brain cancer, it will be important to note whether ATN-224 crosses the blood brain barrier to chelate copper in brain tissue or whether it remains within cerebral vasculature. One study suggests the possibility that ATN-224 can indeed cross the blood brain barrier, based on evidence from Cu-TM complexes and from studies of copper diethyldithiocarbamate, another non-polar lipophilic chelate[56-57]. This is vital in determining whether ATN-224 can prevent the inhibitory effects of copper (II) ions against oHSV at the actual site of the virus's action.

More immediate future studies of this project will aim to determine the effect of ATN-224 on the host immune response. In particular, we would like to examine the inflammatory cytokine response, immune cell infiltration and macrophage activity post-OV treatment in mice treated with or without ATN-224. We would like to assess the effect of ATN-224 on OV persistence, analyzing the amount of virus present in tumors over several time points. Lastly, we would like to evaluate the biodistribution of OV in various organs following intravenous delivery of virus.

This study aimed to test the effects of ATN-224 on the anti-tumor efficacy of oncolytic virus therapy in order to provide a model for the treatment of cancer using anti-angiogenic agents in combination with oncolytic virus. Additional studies should be conducted to evaluate the feasibility of this treatment regimen in combination with

existing treatment modalities, so as to implement an effective therapy in patients with deadly cancers such as malignant glioma.

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